Localized Rabies Infection in Mice[1] (36547)

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The common belief that rabies infection of man and animals is always fatal has been challenged recently by numerous observations. Although it has been known for many years (1, 2) that vampire bats can be carriers of rabies for long periods of time, and are able to transmit infection through bites while remaining symptomless themselves, it was considered a peculiarity of that host. Several authors (3–7) have observed that in large numbers of mice inoculated with rabies virus, a few infected ones remained well, or survived with paralysis, tremors, or general malaise. The term "abortive rabies infection," which was first introduced by Koch (8), was adopted by Bell (6) to describe animals that survived experimental rabies infection after developing symptoms of the disease.

The first well-documented case of rabies infection in man followed by recovery was recently reported (9), that of a 6-year-old boy who was bitten by a rabid bat.

In the course of investigating survival, after inoculation with fixed or street rabies viruses, of mice previously vaccinated with the nonpathogenic Flury HEP strain of rabies, we noted that a number of animals developed a permanent paralysis of the inoculated limb, but survived for periods of over 1 year; this report presents the findings of a study of this phenomenon.

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Materials and Methods. Animals. Swiss white female mice, 4–6 weeks old, were used throughout this study except for the detection and titration of HEP virus, for which newborn animals, 3–4 days old, were used.

Virus strains. Standard challenge virus (CVS), a fixed strain of rabies virus propagated in mouse brain, was obtained from the National Institutes of Health, Bethesda, Maryland. A pool of virus was prepared as a 10% mouse brain suspension in 50% serum–water. Repeated titrations in mice revealed a titer of $10^{7.8}$ LH_{50} by intracerebral inoculation, and $10^{4.0}$ LD_{50} by intraplantar inoculation, per gram of original brain tissue, with an incubation period of 5 days.

The DR strain of street rabies virus was obtained from the Panamerican Center for Zoonosis, Buenos Aires, Argentina. It was originally isolated from a rabies-infected vampire bat (*Desmodus rotundus*) in Brazil and was maintained in mice for 20 consecutive passages. A pool of virus was prepared in mice as a 10% brain suspension in 50% serum–water. The titer of this pool was $10^{7.3}$ LD_{50} by intracerebral inoculation, and $10^{3.9}$ LD_{50} by intraplantar inoculation/g of original brain tissue with an incubation period of 8 days.

The Flury high egg passage (HEP) strain of rabies virus (10) was adapted to growth in the human diploid cell strain (HDCS) WI-38 and propagated in these cells for 16 passages (11). Pools of virus were prepared in BHK-21 cells (12) directly from HDCS seed or after clone purification in agarose-suspended BHK-21/13S cells (13). The titer of these pools was 7.7×10^7 to 1.2×10^8 plaque-forming units (PFU)/ml in agarose-suspended BHK-21/13S cells (13).

Vaccination of animals. Mice were vaccinated with HEP virus by one of the following three routes of inoculation: intracerebral (0.03 ml); intraperitoneal (0.25 ml); or intramuscular, in the gastrocnemius muscle (0.1 ml).

Challenge of animals. Animals were challenged by intraplantar inoculation, according to the method of Krause (14), with 0.03 ml of a virus dilution calculated to contain 10–30 LD_{50}/inoculum.

Irradiation of animals. Mice were exposed to 600 R in a cesium 137 gamma irradiator with a dose rate of 179 R/min, and inoculated immediately after exposure.

Observation of animals. Mice were observed daily for a period of 60 days after the challenge and the number of sick, paralyzed, and dead animals was recorded. Some of the paralyzed mice were kept under observation for over 1 year.

Assay of interferon and virus neutralizing antibody in the serum and brain tissue. Animals were bled, under anesthesia, either by cardiac puncture or by sectioning of cervical blood vessels, and the serum was separated. The brains were harvested aseptically and homogenized in phosphate-buffered saline (PBS) to give a 10% suspension. After centrifugation at 2000 rpm for 15 min, the supernatant fluid was collected and inactivated by heat (56° for 30 min) before neutralizing antibody titration and by acid dialysis, at pH 2, before interferon titration. Sera or brains from 3 to 4 animals were pooled for antibody and interferon determinations.

Interferon. The interferon levels of serum and brain tissue extracts was determined in monolayers of L cells (15) according to the method of Wagner (16) with the Indiana type of vesicular stomatitis virus (VSV) used as an indicator. Each interferon sample was tested in threefold dilutions; the titer of interferon in units per mole is expressed as the reciprocal of the end-point dilution producing a 50% reduction of VSV plaques.

The identity of this viral inhibitor as interferon was confirmed by: (i) its lack of antiviral effect in tissue cultures of heterologous species (chick fibroblasts), (ii) its total inactivation following digestion with trypsin, (iii) its resistance to high acidity (pH 2), and (iv) the fact that the active substance remained in the supernatant fluid after centrifugation at 100,000*g* for 2 hr.

Neutralization test. The plaque reduction assay in agarose-suspended BHK-21/13S cells (17) was used. Each serum sample was tested in serial twofold dilutions reacted with HEP rabies virus suspension containing 1000 PFU/ml. The neutralizing antibody titers are given as the reciprocal of end-point dilutions causing a 50% reduction in plaques.

Histological techniques. Thirteen animals

TABLE I. Sparing Effect of HEP on Mice Challenged[a] with CVS Strain.[b]

HEP inoculation		Condition of mice challenged with CVS		
Time before challenge (days)	Route	Paralysis and death	Paralysis and survival	Survival with no symptoms
8	ic	0	0	10
	ip	1	0	9
6	ic	0	2	8
	ip	4	0	6
4	ic	0	5	5
	ip	6	0	4
2	ic	1	2	7
	ip	7	0	3
1	ic	10	0	0
	ip	10	0	0

[a] By intraplantar route.

[b] ic = intracerebral; ip = intraperitoneal.

were selected for histological study. Sciatic nerves, brains and spinal cords were removed, fixed in formalin, processed in the usual manner, and embedded in paraffin. Sections were stained with hematoxylin and eosin, luxol fast blue-cresyl violet (Klüver-Barerra), and silver according to Bodian's technique.

Results. Virtually all mice inoculated either intracerebrally or intraperitoneally with the HEP Flury strain of rabies virus, and challenged 8 days later with the CVS strain by the intraplantar route, were protected from the lethal effect of CVS (Table I). However, when the period between the first inoculation and the challenge was shortened, the sparing effect of the intracerebrally injected HEP was more pronounced than that of HEP administered intraperitoneally. When the mice received HEP by either route 1 day before challenge, all the animals died.

The sparing effect of intracerebral inoculation of HEP was further substantiated by use of serial tenfold dilutions of the virus in mice challenged 2 days later with CVS. The effect was seen through dilutions of 10^{-2} (Table II). The mice that became paralyzed after challenge with CVS virus were monoplegic, the paralyzed limb being the one inoculated intraplantarly with CVS. The other limbs were never affected and the animals survived 1 year or more after these injections without other complications. In contrast, control mice died within 6–8 days after challenge.

TABLE II. Sparing Effect of Dilutions of HEP Flury on Mice Challenged[a] 2 Days Later with CVS.

HEP dilution[b]	Condition of mice challenged with CVS		
	Paralysis and death	Paralysis and survival	Survival with no symptoms
Undil.[c]	1	3	6
10^{-1}	6	3	1
10^{-2}	6	1	3
10^{-3}	10	0	0

[a] By intraplantar route.
[b] Inoculated by intracerebral route.
[c] Infectivity, 1.2×10^8 PFU/ml.

Recovery of rabies virus from the nervous tissue of inoculated mice. Mice inoculated intracerebrally with HEP were sacrificed daily after inoculation, and the presence of virus in their brain tissue was determined by plaque assay and by inoculation of newborn mice. The concentration of rabies virus per gram of brain was $10^{4.6}$ PFU on the first day after inoculation and $10^{3.3}$ PFU on the second day. No infectious virus was recovered from the brain tissue on or after the fourth day following inoculation. No virus was recovered at any time from the lumbar enlargement of the spinal cord.

Infectious virus could not be recovered from monoplegic animals sacrificed 7 days after challenge with CVS. However, virus could be recovered from the brain, lumbar cords, and sciatic nerves of mice that became either paraplegic or quadriplegic after challenge. The virus recovered appeared to be the CVS strain, as determined by its pathogenicity for adult mice.

Sparing effect of HEP on street virus infection. The results of these studies suggest that centripetal spread of the lethal fixed virus is arrested at the peripheral nerve or low spinal cord level, if the mouse has been inoculated previously with the HEP strain of virus. However, the difference in incubation time between street virus and fixed virus suggested further study.

Two experiments were performed.

In the first experiment, adult mice were given HEP virus by various routes either 2 hr before or 24 hr after intramuscular challenge with street virus (Table III). Whereas parenteral inoculation with HEP virus had little, if any, sparing effect on challenge with street virus, intracerebral inoculation of HEP virus had a marked sparing effect on mice that received it 2 hr before or 24 hr after street virus. In the second experiment (Table III), mice were given only intracerebral inoculations of HEP and were challenged with street virus either 2 hr before or at various times after injection of HEP. All 12 control animals died after challenge with street virus, but intracerebral inoculation with HEP showed a marked protective effect even when

TABLE III. Sparing Effect of HEP on Mice Challenged[a] with Street Virus.[b]

HEP inoculation		Condition of mice challenged with street virus		
Time (hr) before (—) or after (+) challenge	Route	Paralysis and death	Paralysis and survival	Survival with no symptoms
Expt. 1				
—2	ic	1	0	9
	im	9	0	2
+24	ic	5	2	4
	im	7	0	4
Control	—	9	0	2
Expt. 2				
—2	ic	1	3	8
+2	ic	1	5	6
+6	ic	0	4	8
+8	ic	5	2	5
+24	ic	6	5	1
Control	—	12	0	0

[a] By intraplantar route.
[b] ic = intracerebral; im = intramuscular.

HEP was injected 24 hr after street virus (Table III). As noted previously in the studies with fixed virus (Tables I and II), many animals inoculated with HEP and challenged with street virus did not die but became partially paralyzed and remained so for periods of time extending over 1 year. The sparing effect of HEP was less pronounced in mice when injected 24 hr after challenge with street virus, but even in this group only half of the challenged animals died and of the remainder, 5 had persistent paralysis and one showed no signs of illness.

Sparing effect of HEP in irradiated animals. To rule out the possibility that the sparing effect of HEP was mediated only through a rapid antibody response by the inoculated animal, groups of mice were exposed to a single dose of 600 R at the time of intracerebral inoculation with HEP. The irradiated and untreated and control mice were challenged 2 hr later with street virus.

Whereas the infection with street virus was more lethal for irradiated mice than for the untreated animals (Table IV), irradiation hardly altered the sparing effect of HEP on street virus challenge. Irradiated mice died from the exposure to irradiation (irradiation sickness) 18 days after irradiation.

Interferon and virus neutralizing antibody elicited in mice by HEP virus. To investigate the mechanisms of the sparing effect of HEP, mice inoculated intracerebrally with this virus were bled at frequent intervals af-

TABLE IV. Lack of Effect of X-Ray Irradiation of Mice on Sparing Effect of HEP on Street Virus Challenge.[a]

	Condition of mice after inoculation with street virus preceded by:					
	HEP inoculation[b]			None		
Treatment	Paralysis and death	Paralysis and survival	Survival with no symptoms	Paralysis and death	Paralysis and survival	Survival with no symptoms
X-Ray	0	3	9	11	0	1
None	0	1	11	8	0	4

[a] By intraplantar route.
[b] Two hours before challenge.

ter infection, and the levels of interferon and neutralizing antibody were determined in their serum and brain tissue. The results (Table V) indicate that 7 hr after inoculation, interferon was present in serum diluted 1:60, the titer rose to 1:200 at 10 hr after inoculation, and dropped to 1:30 at 24 hr after infection. From the second day, interferon could no longer be detected in the serum of the animals. Interferon was first detected in brain tissue 14 hr after injection, and its concentration increased during the next 2 days, reaching its peak, 1:240, during the third and fourth day after injection. Brain interferon could still be detected on the fifth day, but not on the seventh day after inoculation.

In contrast to the rapid appearance of interferon, a rabies neutralizing antibody was first detected in both serum and brain tissue of the animals on the second day after inoculation with HEP. Thereafter, the titer of the neutralizing antibody in serum and brain tissue rose rapidly, reaching its peak 15 days after virus inoculation and persisting at almost the same level 30 days after infection.

TABLE V. Presence of Neutralizing Antibodies and Interferon Activity in Serum and Brain Tissue of Mice Inoculated Intracerebrally with the HEP.

	Virus neutralizing antibody		Interferon	
Time (hr)	Serum	Brain tissue	Serum	Brain tissue
0	<10	<10	<30	<30
7	<10	<10	60	<30
10			200	<30
14			120	30
18			30	30
24	<10	<10	30	30
(days)				
2	640	160	<30	60
3			<30	240
4	2000	1000	<30	240
5			<30	60
7	6000	2000	<30	<30
15	16,000	8000		
		900[a]		
30	10,000	4000		

[a] After body perfusion.

By perfusing some animals with PBS before harvesting their brains, we demonstrated that the virus neutralizing capacity of the brain tissue reflected the antibody levels in the circulating blood. In the perfused animals, the titer of virus neutralizing antibody was decreased by 90%.

Histological findings. Histological findings may be summarized as follows:

1. Mice sacrificed 2 days after the onset of paralysis caused by injection of street virus showed no histological lesion in the CNS despite the obvious clinical symptoms.

2. Mice sacrificed 4 days after onset of lower-limb paralysis caused by injection of street virus displayed an obvious acute inflammatory reaction, consisting of diffuse activation of microglia with a mild polymorphonuclear cell response and some perivascular cuffing. This was most severe in the spinal cord and diminished markedly in the most rostral portions of the CNS.

3. Mice sacrificed 26 days after the onset of right-leg paralysis following inoculation with HEP and street virus displayed a variety of chronic inflammatory lesions in the affected extremity. These were less intense than those seen in the previous group. The nerves showed loss of myelin and some Schwann cell proliferation. Axis cylinders were relatively intact.

4. No specific changes could be observed in unparalyzed mice inoculated with HEP-CVS virus.

5. Mice with monoplegia of slightly over 1 year's duration also showed nonspecific CNS changes. The peripheral nerves of the affected limb, however, showed striking myelin loss, and in one case at least, this was accomplished by Schwann cell proliferation.

Discussion. Contrary to general belief, not all animals showing signs of sickness after street rabies virus infection die. The anecdotal evidence supporting this statement has recently been supplanted by observation that 16% of laboratory mice infected experimentally through the intraperitoneal route survived after showing signs of sickness (7). In the present study, it was possible to induce, in mice, a chronic infection of the CNS, as evidenced by permanent monoplegia, para-

plegia or quadriplegia. These symptoms were a direct result of inoculation of an otherwise lethal dose of either CVS or street virus and HEP Flury virus which is apathogenic for adult mice (10). The sparing effect of HEP Flury was only observed after the virus was injected by an intracerebral route into mice challenged with either the CVS or street virus. Mice infected with the street virus could be saved from death even when the HEP Flury strain was injected as late as 24 hr after their exposure to street virus. Mice infected with CVS, a virus infection characterized by a relatively short incubation period could be spared only by the administration of HEP Flury virus no later than 2 days before challenge.

The spread of infection by the virulent virus was often arrested by the HEP virus at the level of the inoculated limb, as was evidenced by chronic monoplegia and degeneration of the peripheral nerves. No virulent virus could be recovered from such animals. Conversely, the CVS virus could be isolated from either paraplegic or quadriplegic mice, which were spared by HEP Flury up to 7 days after exposure. These mice showed an acute inflammatory reaction observed in the corresponding segment of the spinal cord.

The sparing effect of the HEP virus was probably not mediated by an antibody response since it was not eliminated by irradiation of the animals with 600 R. Moreover, the fact that neutralizing antibodies could not be detected in either the peripheral or brain circulation of mice exposed to HEP virus earlier than 2 days after inoculation with the virus suggests that mechanisms other than circulating rabies antibody mediates the sparing effect of HEP virus. The rapid induction of circulating interferon (within 7 hr) and brain interferon (within 14 hr) after exposure to HEP suggests that arrest of the spread of the virulent virus may be mediated through interferon, instead.

The observations in monoplegic mice, 1 year or more after onset of sickness, of persistent degeneration of a portion of peripheral nerve, accompanied by Schwann cell proliferation, may be an indication that an active infectious process was still operating in these animals, even though no infectious virus was isolated from them. Thus, in theory at least, an otherwise virulent rabies virus may become involved in a chronic disease state of the CNS in a way similar to that by which other neurotropic agents become involved in the so-called slow virus diseases. Whether a similar situation may develop in a human subject is difficult to state at the present time. The survival of a child after severe rabies infection (9) indicates at least that the dogma of "incurable wound" (18) must undergo review, not only in view of the lives of animals, but also in the case of human subjects.

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